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Full Article

Comparative Study of Three Vaccinal Strains of *Clostridium tetani* Including Harvard 52, G5 and 49205 from Standpoint of Six Essential Factors to Evaluate Their Toxigenesis for Use in Tetanus Vaccine Production

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ABSTRACT

Tetanus is an important disease which created by tetanospasmin toxin of *Clostridium tetani*. In this study we surveyed six important factors including LF, KF, MLD, pH, OD and total protein assay of Harvard 52 (H52), G5 (HG5) and 49205 (H49205) strains of the bacterium to determine which of them were more suitable for use in vaccine production. The mentioned strains were seperatedly reactivated in thioglycolate medium, 2ml of this suspension was used to inoculate the Muller-Miller medium of each of sixteen 500 ml Erlenmeyer flasks (7,6 and 3 flasks for H52, HG5 and H49205 respectively), where the fermentation runs were performed. Over a period of seven days of experiment, several tests for evaluation of the six mentioned factors on samples of medium cultures were carried out. Results revealed that H52 strain had significantly lower values in LF and OD compared to strains HG5 & H49205 ($P<0.001$ and $P<0.01$ respectively) while its MLD and pH were better than other strains ($P< 0.05$ and $P< 0.02$ respectively). In conclusion, it seems that HG5 & H49205 strains have been greater toxin producer than H52 strain and as a result, we hope that with some complementary works, these two strains such as H52 strain, be used for routine tetanus vaccine production.

Keywords: *Clostridium tetani*, Harvard 52, Harvard G5, Harvard 49205, toxigenesis

INTRODUCTION

Tetanus is a life-threatening disease caused by action of a powerful tetanospasmin or tetanus neurotoxin, an exotoxin of gram positive, anaerobic *Clostridium tetani* which can cause death in infected person due to paralysis of respiratory muscles. The bacterium is a common inhabitant of soil, dust, and manure, and can contaminate through abrasion of the skin. The disease cannot be transmitted from person to person (Plourde-Owobi *et al* 2005). Tetanospasmin is a dichain or two-component construction (Middlebrook and Dorland 1984), consists of A and B toxin which is produced as a single chain and then released into the medium after cell lysis. It cleaved by indigenous proteolytic enzymes to a light chain (fragment A) in NH2 terminal and a

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heavy chain (fragment B-C) in COOH terminal polypeptide which attach together by a reducible disulfide bridge. Toxin produced in the infected tissue by the bacteria grown under anaerobic condition, binds through the COOH-terminal fragment C to the gangliosides and proteins of the peripheral nerve terminals (WHO, manual). The A subunit undergoes a retrograde transport *via* the nerve axon to the spinal cord. This highly toxic enzyme specifically cleaves one of the nerve cell proteins, *i.e.*, synaptobrevin, resulting in inhibition of the release of neurotransmitters (glycine and GABA) from inhibitory interneurons causing spastic paralysis, the characteristic of tetanus (Indrawattana 2010). In toxin production by vaccinal strains, some factors such as genetic of strain, evacuation of metabolite gases, temperature, pH regulation and enrichment level of culture medium are much important which should be taken into consideration. To obtain a better seed, medium or other circumstances, researchers have always been interested to increase production of more toxin of *Cl. tetani*. Proteolytic enzymes are factors which may affect toxin activity (Helting *et al* 1979). It's been shown protease activity in various time of bacterial media culture, but the most activity of them were seen when the bacterial mass was in high level. There are three protease which the smallest of them with molecular weight of 27000 Da released to the medium as soon as bacterial lyses, and is the cause of changing tetanospasmin protoxin to active extra form of the toxin which made of A and BC fragments as light and heavy chains respectively (Fairweather *et al* 1987).

It seems that the maximum toxin production occurring in stationary stage of growth, so use of some materials such as glutamate which cause the decrease in time of this stage can also result the decrease in amount of toxin production (Bizzini 1979). Few proteases that produced by *Cl. tetani* may damage to the toxin and decrease its activity. One of the factors which has a profound effect on toxin production, is the kind of vaccinal strain. Hence search for finding a better toxin producing strain is an interest field of researchers. According to present protocols in vaccine production, some factors including LF (Limes flocculation; the amount of toxin or toxoid which when mixed with 1 International Unit of antitoxin gives a Ramon flocculation in the shortest time), KF (Flocculation time in minute, as observed in the flocculation reaction), MLD (minimal lethal dose; the amount of toxin which kills animals within four days) amount of nitrogen (for use in calculating of toxin purity), pH (measure of the acidity or basicity of an aqueous solution) and OD (optical density, absorbance) are very important in evaluation and control of toxin production (RIVM, 1999, WHO manual). In this study we surveyed three vaccinal strains, including strain H52, which is used routinely in production of tetanus vaccine in Razi institute, HG5 and H49205 to understand which of them is more powerful in toxigenesis, on the basis of six important above mentioned factors in relation with two others strains for use in tetanus vaccine mass production.

MATERIALS AND METHODS

Vaccinal strains including: Harvard 52(H52), G5 (HG5) and 49205 (H49205) of the *Cl. Tetani*. Thioglycolate and Muller-Miller medium for reactivation and toxigenesis of the lyophilized *Cl tetani* respectively. Tetanus antitoxin for evaluation of toxin. Peptone solution for serial dilation of toxin for MLD tests. Syringes for injections to experimental animals and NIH laboratory mice for MLD tests.

1- Three lyophilized vaccinal strains of *Cl. tetani* were seeded in fluid thioglycolate medium and incubated at 35 ^{o}C for 24- 48 h and then 2ml of this suspension were used to inoculate the Muller-Miller medium in sixteen 500 ml Erlenmeyer flasks where the fermentation runs were performed. Inoculated flasks were incubated at 35^{o}C for 7 days. Over a period of seven days of experiment, several samples were taken and Gram stain was performed for detection of spontaneous infection.

2- Total of 16 containers (500 ml Erlenmeyer flasks) including, 7, 6 and 3 flasks was allocated for H52, HG5

and H49205 vaccinal strains respectively. Volume of each culture experiment was 200 ml.

3- Metabolite gases produced in flasks of three groups send out by push of mild filtered air flow through two successive 0.2 µm size pore filters, and exhaust air from flasks through a 0.4 µm size pore filter.

4- From second to last day of experiment, some samples were taken in sterile condition and were measured for pH and OD values and also for Gram´s staining when needed. Samples took from fourth to last day were used for measurement of LF and KF values in addition to other previously mentioned tests.

5- MLD test was performed for samples of groups taken in fourth to last day of experiment and were showed valuable values. For performing of the test, we created serial dilution from 1/100000 to 1/5000000 of toxin in %1 peptone solution. Each dilution was injected subcutaneously to two 18-20g NIH mice, and the animals were checked for detection of tetanus toxin intoxication signals, for subsequently 4 days. The last dilution in each serial dilution which caused the death of at least one of two mice in each group, assigned as MLD value of that sample.

6- Total protein assay were performed for some valuable samples with Lowry's method.

7- At the end of experiment, data of experiments compared with t- test analysis method.

RESULTS

1- The average of total samples for LF assay of three vaccinal strains, were 67.86 (H52), 231.67(HG5) and 153.33(H49205) LF/ml, which H52 showed significant difference with two other strains (P< 0.001 and P< 0.01 respectively) (table1),(figure 1).

2- The average of total samples for KF value were 29.86 (H52), 34.83 (HG5) and 34.67 minutes (H49205), so there weren't any significant difference between three groups (P> 0.05).

3- The average of samples for minimal lethal dose (MLD) values were 2.64×10^{-6} (H52), 1.04×10^{-6} (HG5) and 2.33×10^{-5} (H49205), and there were significant difference between H52 with HG5 (P< 0.05) and H49205 (P<0.02), (figure 2).

4- The average of total protein was 12.28 mg/ml for H52; and same amount of 12.2 mg/ml for HG5 and H49205. So there weren't any significant difference between groups.

5- Algebraic sum of alterations pH values of last day (7^{th} day) compared with culture day were - 0.004 (H52), - 0.548 (HG5) and - 0.773 (H49205). So there were significant differences between H52 with HG5 and H49205 (P< 0.05)

6- Finally algebraic sum of alterations OD values of last day (7^{th} day) compared with culture day were, 0.547 (H52), 0.786 (HG5) and 1.184 (H49205). Hence there were significant differences between H52 with HG5 (P< 0.02) and H49205 (P< 0.001) and also between HG5 with H49205 (P< 0.02) (table 1).

DISCUSSION

Tetanus is persecution disease caused by absolute anaerobic *Clostridium tetani* bacterium which has high mortality rate (De Luca *et al* 1997, Hatheway 1990) especially in neonate patients (Demain *et al* 2005, Bruggemann and Gottschalk 2004). Fortunately it may be prevented by toxoid vaccine created by formaldehyde detoxification of tetanospasmin toxin of the bacterium. As the result show, amount of toxin

Table 1. The summary results of three vicinal strains H52, HG5 and H49205

strain	average				algebraic sum of alterations of 0^{th} with 7^{th} day	
	LF (LF/ml)	**KF** (minute)	**MLD** (dilution)	**Total protein** (mg/ml)	**pH**	**OD** (590 nm)
H52	65 [a]	29.86	10^{-6} [d] × 2.64×10^{-6} [d]	12.28	- 0.004 [g]	0.547 [m]
HG5	231.67 [b]	34.83	10^{-6} [e] × 1.04×10^{-6} [e]	12.1	-0.548 [h]	0.786 [n]
H49205	153.33 [c]	34.67	10^{-5} [f] × 2.33×10^{-5} [f]	12.37	-0.773 [k]	1.184 [p]

Footnote: There are significant difference between a with b and c (P< 0.001 and P< 0.01 respectively); between d with e and f (P< 0.05 and P< 0.02 respectively); between g with h and k (P< 0.05); between m with n and p (P< 0.02 and P< 0.001 respectively) and between n with p (P< 0.02). H= Harvard; Lf= limes flocculation; KF= flocculation time; MLD= minimal lethal dose; OD= optimal density

produced by HG5 and H49205 strains was better than the H52 strain with 231.67 and 153.33 LF/ml respectively in comparison to 65 LF/ml for H52 strain, hence a significant difference were seen between these strains and strain H52 (P< 0.001 and P< 0.01 respectively).

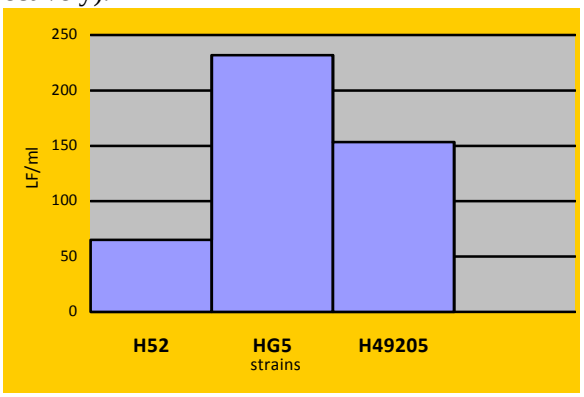

Figure1. Comparison of LF values in three vaccinal strains

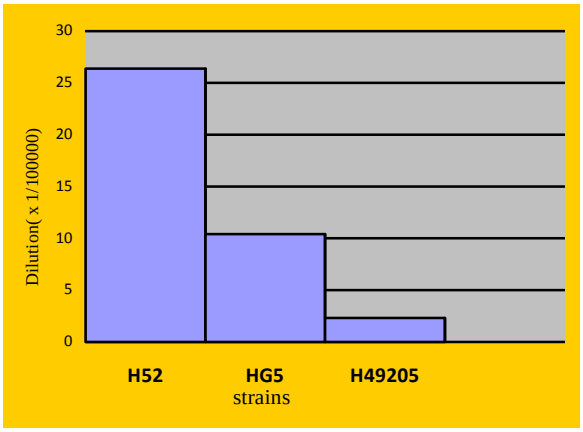

Figure 2. Comparison of MLD values in three vaccinal strains

As a result HG5 was the most toxin producer in comparison with the other two strains which may relate to their genetic background. As this factor has an important role on toxigenesis of the vaccinal strains of *Cl. tetani* (WHO manual) , which may demonstrate its effect via some ways such as, growth rate and proliferation, quantity or quality of toxin production, resistance of toxin to pH variation, and acquired resistance of proteolytic enzymes. In case of H49205 which is a relatively high toxin producer, result of our study wasn't dissimilar to other researchers that found, the new strain which is derived from H49205, named Y, can produce a large amount of toxin (RIVM, 1999). Comparison of pH value of three strains showed that algebraic sum pH value of the first day of culture compare to last day of incubation, were - 0.004, -0.548 and -0.773 for H52, HG5 and H49205 strains respectively, which showed a significant differences between H52 with two other strains (P< 0.05).

It means that, the culture medium was more acidic for HG5 and especially for H49205 strains. Decrease of pH value in culture medium can alter the structure and denaturation of proteins(Bizzini 1979) such as tetanus toxin molecule which in turn may cause reduce interaction with tetanus antiserum , decrease of LF and MLD values and also increase of flocculation time or KF value. Although, in KF values there were not any significant difference, but in MLD values we saw a significant difference between H52 with HG5 and H49205 (P< 0.05 and P< 0.02 respectively). In other words, pH value seems to confirm the reduction of MLD value due to decrees of pH and its effect on structure of toxin, especially this is more obvious for H49205, which its MLD and algebraic sum values are the lowest in comparison with two other strains. However in case of LF value, it seems that, due to high proliferation of HG5 and H49205 strains, and consequently increase toxin production, the harmful effect of pH on the toxin, has not been seen. Comparison of OD values of three strains revealed that H49205 was had the much higher OD than the other strains and a significant difference were seen with HG5 and H52 strains (P< 0.02 and p< 0.001 respectively). It means that impact cells were produced a large amount of acidic materials and consequently decrease of medium pH, that in turn cause of damage or denaturation of toxin or activity of indigenous enzymes for changing of inactive protoxin to active tetanus extracellular toxin. For release of toxin to the culture medium, the bacterium should be lysed by autolytic enzymes that are present in the cell wall of *Cl. tetani* (Neubauer & Torsten 1981, Bizzini 1979). Conversion of intracellular toxin, as a protoxin devoid of significant toxicity by itself, into biologically active extracellular

form of the toxin is performe by protease enzymes via nicking of the toxin polypeptide chain (Bizzini 1979), however in the strains which their LF values were lower than the other, it may be cell lysis occurs in lower frequency or it may be damage to their proteolytic bacterial enzymes.

The average of MLD value for H52 strain was 2.64×10^{-6} while for HG5 and H49205 strains were 1.04×10^{-6} and 2.33×10^{-5} respectively, which showed a significant difference with both of them ($P< 0.05$ and $P<0.02$ respectively). With denaturation of toxin because of reduced pH value during the growth rate of culture, it may be toxin somewhat became unable to recognize and appropriately binded to its receptors on neuron cells, hence the experimental animals less injured, and the MLD test value become weaker than the other MLD tests. In general, it seems that toxigenesis of HG5 and H49205 strains were better than the H52 strain, while having a KF and MLD values weaker than the H52 strain, that may due to lower pH value during the bacterial cultivation, which in turn damages the toxin and deleterious of these factors. We hope with some of complementary works be able to use HG5 and H49205 strains instead of H52 strain, for routine tetanus vaccine mass production.

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References

Bizzini, B. (1979). Tetanus toxin. *Microbiological Reviews* June: 224-240.

Bruggemann, H., Gerhard, G. (2004).Insights in metabolism and toxin production from the complete genome sequence of Clostridium tetani. *Anaerobe* 10: 53–68.

De Luca, M.M., Abeiro, H.D., Bernagozzi, J.A., Basualdo, J.A.(1997). Nitrogen-gas bubbling during the cultivation of Clostridium tetani produces a higher yield of tetanus toxin for the preparation of its toxoid. *Microbiology Immunol*ogy 41(2):161-3.

Demain, A. L., Donald, F. Fang G., A. (2005). Effective levels of tetanus toxin can be made in a production medium totally lacking both animal (e.g., brain heart infusion) and dairy proteins or digests (e. g., casein hydrolysates) *Vaccine* 23: 5420 – 5423.

Fairweather, N. F., Lyness, V. A. and Maskell D. J. (1987). Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *E. coli*. *Infection and immunity* November: 2541-2545.

Hatheway, C. L. (1990). Toxigenic Clostridia. *Clinical Microbiology Reviews* January: 66-98.

Helting, T.B., Parschat, S. and Engelhardt, H. (1979). Structure of Tetanus Toxin. Demonstration and separation of a specific enzyme converting intracellular tetanus toxin to the extracellular form. *The Journal of Biological Chemistry* 254(21), November 10: 10728-10733.

Indrawattana, N., Sookrung, N., Kulkeaw, K., Seesuay, W., Kongngoen, T., Chongsa-nguan, M., Tungtrongchitr, A. and Chaicumpa W. (2010). Human Monoclonal ScFv That Inhibits Cellular Entry and Metalloprotease Activity of Tetanus Neurotoxin. *Asian pacific journal of allergy and immunology* 28: 85-93.

Middlebrook, J. L. and Dorland, R. B. (1984). Bacterial toxins: cellular mechanisms of action. *Microbiological Review* September: 199-221.

Neubauer, V. and Torsten, B. H. (1981). Structure of Tetanus Toxin. The arrangement of papain digestion products within the heavy chain framework of extracellular toxin. *Biochemica et Biophysica Acta* 668: 141-148**.**

Plourde-Owobi L., Seguin, D., Baudin,M.A., Moste, C., and Rokbi, B.(2005). Molecular Characterization of *Clostridium tetani* Strains by Pulsed-Field Gel Electrophoresis and Colony PCR. *Applied Environmental Microbiol*ogy September 71(9): 5604–5606

RIVM/BIS/VTTC. (1999), Chapter3. Strains and seed lots. 231.

WHO, manual for the production and control of vaccines tetanus toxoid.) BLG/UNDP/77.2, Rev.1